JC10 Rec'd PCT/PTO 01 FEB 2002

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR)
112843-041	10/049368

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/07207	26 July 2000	05 August 1999

TITLE OF INVENTION
BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA

APPLICANT(S) FOR DO/EO/US
Bruessow et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Indications Relating to Deposited Microorganism or Other Biological Material, Express Mail No.: EL727382069US, return receipt postcard

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/049368	PCT/EP00/07207	112843-041

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	21 - 20 =	1	x $18 00	**$18.00**	
Independent claims	4 - 3 =	1	x $84.00	**$84.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
	TOTAL OF ABOVE CALCULATIONS =			**$992.00**	
☐	Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.			**$0.00**	
	SUBTOTAL =			**$992.00**	
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +			**$0.00**	
	TOTAL NATIONAL FEE =			**$992.00**	
☐	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			**$0.00**	
	TOTAL FEES ENCLOSED =			**$992.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$992.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

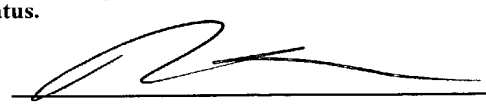
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-1818** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Robert M. Barrett (Reg. No. 30,142)
Bell, Boyd & Lloyd LLC
P.O. Box 1135
Chicago, Illinois 60690
312-807-4204



SIGNATURE

Robert M. Barrett
NAME

30,142
REGISTRATION NUMBER

February 1, 2002
DATE

10/049368
JC13 Rec'd PCT/PTO 01 FEB 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Bruessow et al.
Appl. No.: PCT/EP00/07207
Filed: Filed Herewith
Title: BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA
Art Unit: Unknown
Examiner: Unknown
Docket No.: 112843-041

Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified patent application as follows:

In the Specification

Please cancel line 1 at page 1 and substitute with the following:

SPECIFICATION

TITLE OF THE INVENTION

"BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA"

BACKGROUND OF THE INVENTION

On page 3, beginning at line 5, please substitute the paragraph with the following:

A need, therefore, exists to provide additional lactic acid bacteria that may exert beneficial activities to living beings upon ingestion.

On page 3, at line 7, please add the following:

SUMMARY OF THE INVENTION

On page 3, beginning at line 14, please substitute the paragraph with the following:

In an embodiment, the Bifidobacteria to be used are preferably selected from the group consisting of Bifidobacterium adolescentis or Bifidobacterium longum, preferably Bifidobacterium adolescentis, and is more preferably Bifidobacterium CNCM 1-2168.

On page 3, beginning at line 25, please substitute the paragraph with the following:

In an embodiment, the present invention also provides for a food or pharmaceutical composition containing at least one of the Bifidobacterium strains capable to essentially prevent infection of intestinal cells by rotaviruses.

On page 4, at line 21, please insert the following:

Additional features and advantages of the present invention are described in, and will be apparent from, the following Detailed Description of the Invention and the figures.

On page 4, at line 22, please delete the text and insert the following:

BRIEF DESCRIPTION OF THE FIGURES

On page 4, at line 26, please insert the following:

DETAILED DESCRIPTION OF THE INVENTION

On page 6, at line 16, please substitute with the following:

The present invention will now be described by way of example and not limitation.

On page 9, at line 20, please insert the following:

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its intended advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

In the Claims:

Please cancel Claims 1 - 9 without prejudice or disclaimer.

Please add newly-submitted Claims 10 – 30 as follows:

10. A method of treating diarrhea, the method comprising administering to a mammal at risk of diarrhea an amount of a lactic acid bacterium of genus Bifidobacterium effective to prevent infection of intestinal cells of the mammal due to a rotavirus.

11. The method according to Claim 10 wherein the Bifidobacterium is selected from the group consisting of Bifidobacterium longum and Bifidobacterium adolescentis.

12. The method according to Claim 10 wherein the Bifidobacterium comprises Bifidobacterium CNCM I-2168.

13. The method according to Claim 10 wherein the Bifidobacterium is administered via an ingestible carrier.

14. The method according to Claim 13 wherein the ingestable carrier includes the Bifidobacterium in an amount ranging from about 10^5 cfu/g to about 10^{12} cfu/g.

15. The method according to Claim 13 wherein the ingestible carrier comprises a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milk,

milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formula and pet food.

16. A method of preventing infection of intestinal cells of a mammal at risk of infection due to a rotavirus, the method comprising administering to the mammal a composition including a therapeutically effective amount of a Bifidobacterium strain.

17. The method according to Claim 16 wherein the composition includes about 10^5 cfu/g to about 10^{12} cfu/g of the Bifidobacterium strain.

18. The method according to Claim 16 wherein the composition comprises a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milk, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formula and pet food.

19. The method according to Claim 16 wherein the composition comprises a pharmaceutical composition selected from the group consisting of a tablet, a liquid bacterial suspension, a dried oral supplement, a wet oral supplement, a dry tube feeding and wet tube feeding.

20. The method according to Claim 16 wherein the Bifidobacterium strain is selected from the group consisting of Bifidobacterium longum, Bifidobacterium adolescentis, and Bifidobacterium CNCM I-2168.

21. A food composition comprising one or more Bifidobacterium strains capable of preventing infection of intestinal cells in mammal due to a rotavirus.

22. The food composition according to Claim 21 wherein the food composition is selected from the group consisting of milk, yogurt, curd, cheese, fermented milk, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formula and pet food.

23. The food composition according to Claim 21 wherein the food composition includes about 10^{11} cfu/g or less of the Bifidobacterium strains.

24. The food composition according to Claim 21 wherein the food composition includes about 10^{5} cfu/g to about 10^{11} cfu/g of the Bifidobacterium strains.

25. The food composition according to Claim 21 wherein the Bifidobacterium strains are selected from the group consisting of Bifidobacterium longum, Bifidobacterium adolescentis, and Bifidobacterium CNCM I-2168.

26. A pharmaceutical composition comprising one or more Bifidobacterium strains capable of preventing infection of intestinal cells of a mammal due to a rotavirus.

27. The pharmaceutical composition according to Claim 26 wherein the pharmaceutical composition is selected from the group consisting of a tablet, a liquid bacterial suspension, a dried oral supplement, a wet oral supplement, a dry tube feeding and wet tube feeding.

28. The pharmaceutical composition according to Claim 26 wherein the pharmaceutical composition includes about 10^{12} cfu/g or less of the Bifidobacterium strains.

29. The pharmaceutical composition according to Claim 26 wherein the pharmaceutical composition includes about 10^7 cfu/g to about 10^{11} cfu/g of the Bifidobacterium strains.

30. The pharmaceutical composition according to Claim 26 wherein the Bifidobacterium strains are selected from the group consisting of Bifidobacterium adolescentis, Bifidobacterium longum and Bifidobacterium CNCM I-2168.

REMARKS

This Preliminary Amendment is submitted in the above-identified patent application. Pursuant to the Preliminary Amendment, Claims 1-9 have been canceled and newly-submitted Claims 10- 30 have been added. This Preliminary Amendment does not add new subject matter. Applicants also note for the record that the purpose of this Preliminary Amendment is to place the claims in proper format and/or add new claims. Therefore, Applicants do not intend to disclaim any subject matter in view of this Preliminary Amendment.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Versions with Markings to Show Changes Made.**"

Respectfully submitted,

BELL, BOYD & LLOYD LLC

BY _____

Robert M. Barrett
Reg. No. 30,142
P.O. Box 1135
Chicago, Illinois 60690-1135
Phone: (312) 807-4204

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Please cancel line 1 at page 1 and substitute with the following:

SPECIFICATION

TITLE OF THE INVENTION

"BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA"

BACKGROUND OF THE INVENTION

On page 3, beginning at line 5, please substitute the paragraph with the following:

~~Consequenty, the problem underlying the present invention~~ is A need, therefore, exists to provide additional lactic acid bacteria that may exert beneficial activities to living beings upon ingestion.

On page 3, at line 7, please add the following:

SUMMARY OF THE INVENTION

On page 3, beginning at line 14, please substitute the paragraph with the following:

~~The~~ In an embodiment, the Bifidobacteria to be used are preferably selected from the group consisting of Bifidobacterium adolescentis or Bifidobacterium longum, preferably Bifidobacterium adolescentis, and is more preferably Bifidobacterium CNCM 1-2168.

On page 3, beginning at line 25, please substitute the paragraph with the following:

~~The~~ In an embodiment, the present invention also provides for a food or pharmaceutical composition containing at least one of the Bifidobacterium strains capable to essentially prevent infection of intestinal cells by rotaviruses.

On page 4, at line 21, please insert the following:

Additional features and advantages of the present invention are described in, and will be apparent from, the following Detailed Description of the Invention and the figures.

On page 4, at line 22, please delete the text and insert the following:

BRIEF DESCRIPTION OF THE FIGURES

On page 4, at line 26, please insert the following:

DETAILED DESCRIPTION OF THE INVENTION

On page 6, at line 16, please substitute with the following:

The present invention will now be described by way of example and not limitation.

On page 9, at line 20, please insert the following:

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its intended advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

In the Claims:

Please cancel Claims 1-9 without prejudice or disclaimer.

Please add newly-submitted Claims 10 – 30 as follows:

10. A method of treating diarrhea, the method comprising administering to a mammal at risk of diarrhea an amount of a lactic acid bacterium of genus Bifidobacterium effective to prevent infection of intestinal cells of the mammal due to a rotavirus.

11. The method according to Claim 10 wherein the Bifidobacterium is selected from the group consisting of Bifidobacterium longum and Bifidobacterium adolescentis.

12. The method according to Claim 10 wherein the Bifidobacterium comprises Bifidobacterium CNCM I-2168.

13. The method according to Claim 10 wherein the Bifidobacterium is administered via an ingestible carrier.

14. The method according to Claim 13 wherein the ingestable carrier includes the Bifidobacterium in an amount ranging from about 10^5 cfu/g to about 10^{12} cfu/g.

15. The method according to Claim 13 wherein the ingestible carrier comprises a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milk, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formula and pet food.

16. A method of preventing infection of intestinal cells of a mammal at risk of infection due to a rotavirus, the method comprising administering to the mammal a composition including a therapeutically effective amount of a Bifidobacterium strain.

17. The method according to Claim 16 wherein the composition includes about 10^5 cfu/g to about 10^{12} cfu/g of the Bifidobacterium strain.

18. The method according to Claim 16 wherein the composition comprises a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milk, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formula and pet food.

19. The method according to Claim 16 wherein the composition comprises a pharmaceutical composition selected from the group consisting of a tablet, a liquid bacterial suspension, a dried oral supplement, a wet oral supplement, a dry tube feeding and wet tube feeding.

20. The method according to Claim 16 wherein the Bifidobacterium strain is selected from the group consisting of Bifidobacterium longum, Bifidobacterium adolescentis, and Bifidobacterium CNCM I-2168.

21. A food composition comprising one or more Bifidobacterium strains capable of preventing infection of intestinal cells in mammal due to a rotavirus.

22. The food composition according to Claim 21 wherein the food composition is selected from the group consisting of milk, yogurt, curd, cheese, fermented milk, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formula and pet food.

23. The food composition according to Claim 21 wherein the food composition includes about 10^{11} cfu/g or less of the Bifidobacterium strains.

24. The food composition according to Claim 21 wherein the food composition includes about 10^5 cfu/g to about 10^{11} cfu/g of the Bifidobacterium strains.

25. The food composition according to Claim 21 wherein the Bifidobacterium strains are selected from the group consisting of Bifidobacterium longum, Bifidobacterium adolescentis, and Bifidobacterium CNCM I-2168.

26. A pharmaceutical composition comprising one or more Bifidobacterium strains capable of preventing infection of intestinal cells of a mammal due to a rotavirus.

27. The pharmaceutical composition according to Claim 26 wherein the pharmaceutical composition is selected from the group consisting of a tablet, a liquid bacterial suspension, a dried oral supplement, a wet oral supplement, a dry tube feeding and wet tube feeding.

28. The pharmaceutical composition according to Claim 26 wherein the pharmaceutical composition includes about 10^{12} cfu/g or less of the Bifidobacterium strains.

29. The pharmaceutical composition according to Claim 26 wherein the pharmaceutical composition includes about 10^7 cfu/g to about 10^{11} cfu/g of the Bifidobacterium strains.

30. The pharmaceutical composition according to Claim 26 wherein the Bifidobacterium strains are selected from the group consisting of Bifidobacterium adolescentis, Bifidobacterium longum and Bifidobacterium CNCM I-2168.

10/049368
PCT/EP00/07207
PTO/PCT Rec'd 01 FEB 2002

Bifidobacteria capable of preventing diarrhea

The present invention pertains to the use of non-pathogenic microorganisms of the genus Bifidobacterium for preparing a carrier for the treatment or prophylaxis of diarrhea brought about by rotaviruses, and to food or pharmaceutical compositions containing such micro-organisms.

Organisms that produce lactic acid as a major metabolic component have been known for a long time. These bacteria may be found in milk or in milk processing factories, respectively, living or decaying plants but also in the intestine of man and animals. These microorganisms, summarized under the term "lactic acid bacteria", represent a rather inhomogeneous group and comprise e.g. the genera Lactococcus, Lactobacillus, Streptococcus, Bifidobacterium, Pediococcus etc..

Lactic acid bacteria have been utilized as fermenting agents for the preservations of food taking benefit of a low pH and the action of fermentation products generated during the fermentative activity thereof to inhibit the growth of spoilage bacteria. In addition, lactic acid bacteria have also been used for preparing from milk a variety of different foodstuff such as cheese, yogurt and other fermented dairy products.

Quite recently, lactic acid bacteria have attracted a great deal of attention in that some strains have been found to exhibit valuable properties to man and animals upon ingestion. In particular, specific strains of Lactobacillus or Bifidobacterium have been found to be able to colonize the intestinal mucosa and to assist in the maintenance of the well-being of man and animal.

In this respect, EP 0 768 375 discloses specific strains of the genus Bifidobacterium, that are capable to become implanted in the intestinal flora and may adhere to intestinal cells. These

Bifidobacteria are reported to assist in immunomodulation, being capable to competitively exclude adhesion of pathogenic bacteria to intestinal cells, thus assisting in the maintenance of the individual's health.

During the last few years research has also focused on the potential use of lactic acid bacteria as probiotic agents. Probiotics are considered to be viable microbial preparations which promote the individual's health by preserving the natural microflora in the intestine. A microbial preparation may be commonly accepted as a probiotic in case the effectual microbes thereof and their mode of action are known. Probiotics are deemed to attach to the intestine's mucosa, colonize the intestinal tract and likewise prevent attachment of harmful microorganisms thereon. A crucial prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

In this respect, WO 97/00078 discloses a specific strain, termed Lactobacillus GG (ATCC 53103), as such a probiotic. The microorganism is particularly employed in a method of preventing or treating food induced hypersensitivity reactions in that it is administered to a recipient together with a food material that has been subjected to a hydrolysis treatment with pepsin and/or trypsin. The Lactobacillus strain selected is described as exhibiting adhesive and colonizing properties and showing a protease enzyme system, so that the protein material contained in the foodstuff to be administered is further hydrolysed by means of proteases secreted by the specific Lactobacillus strain. The method discussed in this document shall eventually result in the uptake of protein material by the gut that does not show a substantial amount of allergenic material anymore.

Further, in EP 0 577 903 reference is made to the use of such lactic acid bacteria having the ability of replacing Heliobacter pylori, the acknowledged cause for the development of ulcer, in the preparation of a support intended for the therapeutic or prophylactic treatment of an ulcer associated with the action of Heliobacter pylori.

In view of the valuable properties particular strains of lactic acid bacteria may exhibit there is a desire in the art to find additional properties of bacterial strains beneficial to the well being of man and/or animal.

Consequently, the problem underlying the present invention is to provide additional lactic acid bacteria that may exert beneficial activities to living beings upon ingestion.

In the course of the studies leading to the invention it was now surprisingly found that microorganisms of the genus Bifidobacterium show properties not yet recognized in the art. In effect, the present invention provides for the use of microorganisms belonging to the genus Bifidobacterium and being capable to essentially prevent infection of intestinal cells by rotaviruses for the preparation of a carrier for the treatment or prophylaxis of diarrhea.

The Bifidobacteria to be used are preferably selected from the group consisting of Bifidobacterium adolescentis or Bifidobacterium longum, preferably Bifidobacterium adolescentis, and is more preferably Bifidobacterium CNCM I-2168.

The microorganisms may be used for the preparation of a variety of ingestable carriers, such as e.g. milk, yogurt, curd, fermented milks, milk based fermented products, fermented cereal based products, milk based powders, infant formulae or pet food and may be included in the respective carrier in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g. For the purpose of the present invention the abbreviation cfu shall designate a "colony forming unit" that is defined as number of bacterial cells as revealed by microbiological counts on agar plates.

The present invention also provides for a food or a pharmaceutical composition containing at least one of the Bifidobacterium strains capable to essentially prevent infection of intestinal cells by rotaviruses.

For preparing a food composition according to the present invention at least one of the Bifidobacterium strains used according to the present invention is incorporated in a suitable

support, in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g, preferably from about 10^6 cfu / g to about 10^{10} cfu / g, more preferably from about 10^7 cfu / g to about 10^9 cfu / g.

In case of a pharmaceutical preparation the product may be prepared in form of tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding or a wet tube feeding etc., with the amount of Bifidobacterium strains to be incorporated therein being in the range of up to 10^{12} cfu / g, preferably from about 10^7 cfu / g to about 10^{11} cfu / g, more preferably from about 10^7 cfu / g to about 10^{10} cfu / g.

The microorganisms may further be formulated in the carrier so as to obtain a desired release pattern, such as encapsulation etc. Based upon the desired objective the person skilled in the art will select the appropriate excipients and/or additives.

The activity of the microorganisms in the individual's intestine is of course dose dependent. That is, the more the microorganisms are incorporated by means of ingesting the above food material or the pharmaceutical composition, respectively, the higher the protective and/or curing activity thereof. Since the used microorganisms are not detrimental to mankind and animals and have eventually been isolated from a natural surrounding, namely baby feces, a high amount thereof may be incorporated so that essentially a high proportion of the individual's intestine will be colonized by the microorganisms.

In the figures,

Fig. 1 shows a scheme illustrating the cell culture screening for assessing rotaviral protective properties of bacterial strains.

During the extensive studies leading to the present invention the inventors have investigated different bacterial strains isolated from baby feces or obtained from the American Tissue and Cell Collection (ATCC 15704). The different strains were examined for their capability to prevent infection of intestinal cells with rotaviruses that are known to cause diarrhea.

Several bacterial genera comprising Bifidobacterium, Lactococcus, Streptococcus were screened for their rotavirus inhibitory properties. The tests for the inhibitory property were essentially performed with three rotavirus serotypes representing the major etiological agents of human viral diarrhea (serotypes G1, G3 and G4).

The various lactic acid bacteria were grown in a suitable medium, such as MRS, Hugo-Jago or M17 medium at temperatures of from about 30 to 40°C corresponding to their optimal growth temperature. After reaching stationary growth the bacteria were collected by centrifugation and re-suspended in physiological NaCl solution. Between the different tests the bacterial cells were stored frozen (-20°C).

The various rotavirus stocks were prepared by infection of confluent cell monolayers. The rotaviruses were incubated before infection. The cells were infected with 20 tissue culture infectious doses.

For assessing anti-rotaviral properties two different protocols were applied. According to one protocol the various bacterial strains were examined for their direct interaction with the rotavirus strain while in the second protocol the bacteria were screened for those strains that interact with cellular rotavirus receptors.

The first protocol involved contacting the respective bacterial suspension each with a different rotavirus strain and incubating in suitable media. Subsequently, the virus-bacteria mixture was applied to a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 (intestinal epithelial cell line) and incubation was continued. Virus replication was then assayed.

The second protocol involved incubating the respective bacterial suspension first together with a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 and adding the virus subsequently. After continued incubation virus replication was assayed.

Rotavirus replication may easily be assessed by histo-immunological staining of rotavirus proteins in infected cells.

A rotavirus inhibitory effect was attributed to a given bacterium when the number of infected cells was reduced by 90% in the cell culture inoculated with rotavirus plus the indicated bacteria in comparison with cells inoculated only with rotavirus.

Out of a total of 260 different bacterial strains primarily isolated merely 4 could be shown to essentially inhibit rotaviral replication. The different bacteria were ascertained to belong to the genus Bifidobacterium subspecies adolescentis or longum. One strain belonging to the species Bifidobacterium adolescentis, which has been termed Bad4, has been deposited in accordance with the Budapest Treaty and has received the deposit number CNCM I-2168. This strain proved to be extremely effective in preventing infection of human cells by rotaviruses.

The present invention will now be described by way of example.

Media and solutions:

MRS (Difco),

Hugo-Jago (Tryptone Difco 30 g/l, Yeast Extract Difco 10 g/l, Lactose Difco 5 g/l, KH_2PO_4 5 g/l, Beef Extract Difco 2 g/l, agar Difco 2 g/l)

M17 (Difco)

M199 (Seromed)

Ringer solution (Oxoid)

PBS (NaCl 8g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l))

Tryptose phosphate broth (Flow)

Trypsin-EDTA solution (Seromed)

Human rotavirus Wa (G1 serotype) and simian rotavirus SA-11 (G3 serotype) were obtained from P.A. Offit, Children's Hospital of Philadelphia, U.S.A. The DS-1xRRV reassortant

virus was obtained from A. Kapikian, NIH Bethesda, U.S.A. The serotype 4 human rotavirus Hochi was obtained from P. Bachmann, University of Munich, Germany.

Example 1

Isolation of lactic acid bacteria from baby feces

Fresh feces were harvested from diapers of 16 healthy babies 15 to 27 days old. 1 g of fresh feces was placed under anaerobic conditions for transportation to the laboratory and microbiological analyses were run within 2 hours from sampling by serial dilutions in Ringer solution and plating on selective media. MRS agar plus antibiotics (phosphomycine 80 µg/ml, sulfamethoxazole 93 µg/ml, trimethoprime 5µg/ml) incubated at 37°C for 48 hours was used to isolate lactic acid bacteria. Colonies were randomly picked up and purified. Physiological and genetic characterisation was performed on the isolates. In the tests another strain obtained from ATCC (ATCC 15704) was also used, which corresponds to the preferred strain Bad4 to be used according to the present invention.

Example 2

Testing of strains in cell culture for anti-rotaviral activity

Several bacterial genera comprising Bifidobacterium, Lactococcus and Streptococcus were selected and tested for members which showed anti-rotaviral activity in the cell culture inhibition test (see below 1st and 2nd protocol). The genus Lactococcus was represented by a single species (Lc. lactis) consisting of two subspecies (Lc. lactis supsp. lactis and cremoris). A total of 30 strains were tested. The Streptococcus genus was represented by a single species (S. thermophilus) with 45 strains. The Leuconostoc and Propionibacterium genus were only represented by a single species (6 strains), while the Enterococcus and Staphylococcus genus was represented by two species each and a total of 17 strains.

In total, 260 bacterial strains were tested for rotavirus inhibitory activity.

1st protocol:

30 µl of the respective bacterial suspension (containing on average $3x10^6$ bacteria) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) to which were added 100 µl of virus in supplemented M199 medium. The virus–bacteria mixture thus obtained was incubated for 1 hour at 4°C and for 1 hour at 37°C. Separately, cells of the human undifferentiated colon adenoma cells HT-29 growing as a confluent monolayer in 96-well microtiter plates (in M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) 1 : 4 diluted with PBS) were washed three times with phosphate-buffered saline (PBS ; pH 7.2). The virus-bacteria mixture processed as indicated above was transferred to the cells and the microtiter plates were incubated for 18 h in a CO_2 incubator (Heraeus). Virus replication was assayed as described below.

2nd protocol:

30 µl of the bacterial suspension (supra) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) and applied directly on HT-29 cells grown and pretreated as described in the 1st protocol in the microtiter plates. After one hour incubation at 37°C 100 µl of virus in supplemented M199 medium were added to the cells in the microtiter plates. The incubation was continued for 18 h in a CO_2 incubator (Heraeus). Virus replication was assayed as described below.

The rotavirus replication was assessed by histo-immunological staining of rotavirus proteins in infected cells as described hereafter.

One day after infection, the cell culture medium was removed from the microtiter plates and the cells were fixed with absolute ethanol for 10 min. Ethanol was discarded, and the plates were washed three times in PBS buffer. Then 50 µl of an anti-rotavirus serum (mainly directed against VP6 protein), produced in rabbits (obtained from the ISREC University of Lausanne) and diluted 1 :2000 in PBS was added to each well and incubated for 1 h at 37°C with a cover slip to prevent desiccation of the wells. The anti-serum was discarded afterwards

and the plates were washed three times with PBS. Then 50 µl of anti-rabbit immunoglobulin G (IgG) antiserum produced in goats and coupled to peroxidase (GAR-IgG-PO; Nordic) were added at a dilution of 1 : 500 in PBS to each well and the plates were incubated for 1 hour at 37 °C. The serum was discarded and the plates were again washed three times with PBS. Then 100 µl of the following substrate mixture was added to each well : 10 ml of 0.05 M Tris-hydrochloride (pH 7.8), 1 ml of H_2O_2 (30% suprapur, diluted 1 :600 in H_2O ; Merck) and 200 µl of 3-amino-9-ethylcarbazole (0.1 g/10 ml of ethanol stored in 200 µl aliquots at – 80 °C ; A-5754 ; Sigma). The plates were incubated for at least 30 min at room temperature. The substrate was discarded and the wells were filled with 200 µl of H_2O to stop the reaction. Infected cell foci were counted with an inverted microscope (Diavert ; Leitz).

Only very few bacterial strains interacted with rotaviruses. Merely 4 out of the 260 bacterial cells primarily selected inhibited rotavirus replication in at least one protocol. Bifidobacterium adolescentis CNCM I-2168 (Bad4) showed an extremely high activity against Serotype 1 Rotavirus, Serotype 3 rotavirus SA-11 and Serotype 4 rotavirus Hochi.

Bad4 is gram positive and catalase negative, it does not produce CO_2 during fermentation and produces just L (+) lactic acid according to methods disclosed in the "Genera of lactic acid bacteria", Ed. B.J.B. Wood and W.H. Holzapfel, Blackie A&P.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 3, line 16.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
Collection Nationale de Cultures de Microorganismes
Institut Pasteur

Address of depositary institution *(including postal code and country)*
25, Rue du Docteur Roux
F-75724 Paris Cedex 15

Date of deposit	Accession Number
15/03/1999	NCC 251 - I-2168

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer
Mrs. H. Fransz

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

Claims

1. Use of a lactic acid bacterium belonging to the genus Bifidobacterium capable of preventing infection of intestinal cells by rotaviruses for the preparation of a carrier for the treatment or prophylaxis of diarrhea.

2. The use according to claim 1, wherein the Bifidobacterium is selected from the group consisting of Bifidobacterium longum or Bifidobacterium adolescentis.

3. The use according to claim 1, wherein the Bifidobacterium is Bifidobacterium CNCM I-2168.

4. The use according to any of the preceding claims, wherein the Bifidobacterium is contained in an ingestable carrier.

5. The use according to claim 5, wherein the Bifidobacterium is contained in the carrier in an amount from about 10^5 cfu / g to about 10^{12} cfu / g carrier.

6. The use according to any of the claims 4 or 5, wherein the carrier is a food composition selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae or pet food.

7. Food or pharmaceutical composition containing at least one Bifidobacterium strain capable of preventing infection of intestinal cells by rotaviruses.

8. The composition according to claim 7, which is selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, pet food, tablets, liquid bacterial suspensions, dried oral supplement, wet oral supplement, dry tube feeding or wet tube feeding.

9. The composition according to claim 7, which is in form of a tablet, liquid bacterial suspension, dried oral supplement, wet oral supplement, dry tube feeding or a wet tube feeding.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2001 (15.02.2001)

PCT

(10) International Publication Number
WO 01/10453 A2

(51) International Patent Classification[7]: A61K 35/74

(21) International Application Number: PCT/EP00/07207

(22) International Filing Date: 26 July 2000 (26.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
99115501.1 5 August 1999 (05.08.1999) EP

(71) Applicant *(for all designated States except US)*: **SOCIETE DES PRODUITS NESTLE S.A.** [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BRUESSOW, Harald** [DE/CH]; Chemin de la Chaumény 13, CH-1814 La Tour-de-Peilz (CH). **RENIERO, Roberto** [IT/CH]; 24, chemin Paudille, CH-1801 Le Mont-Pelerin (CH). **SIDOTI, Josette** [IT/CH]; Route du Lac 24, CH-1094 Paudex (CH).

(74) Agent: **STRAUS, Alexander**; Becker, Kurig, Straus, Bavariastrasse 7, D-80336 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette

WO 01/10453 A2

(54) Title: BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA

(57) Abstract: The present invention pertains to the use of microorganisms belonging to the genus Bifidobacterium for preparing a carrier for the treatment or prophylaxis of diarrhea. The invention also relates to food or pharmaceutical compositions containing such microorganisms.

10/049368

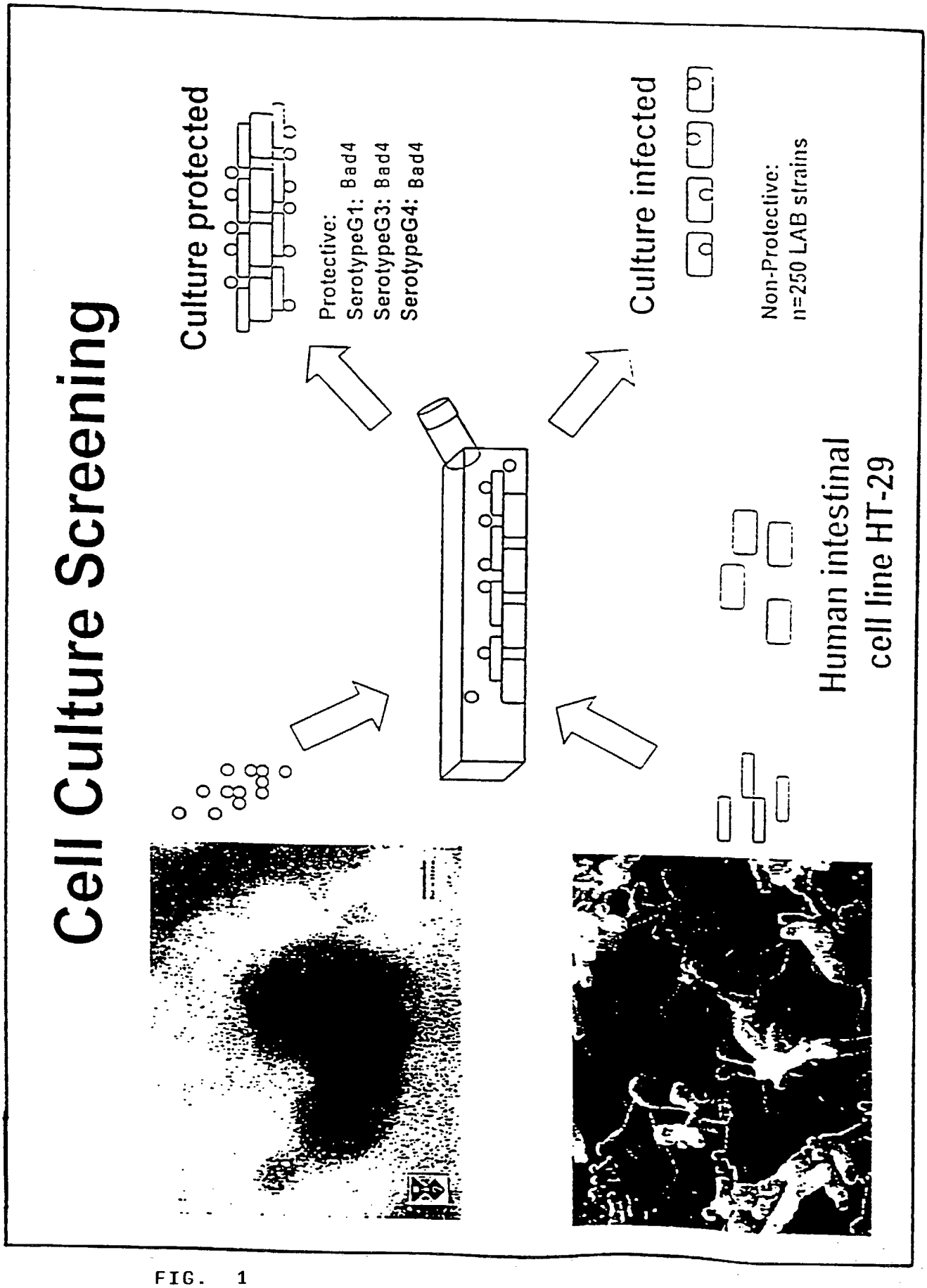


FIG. 1

6538/US 80188/US

Docket No. 112843-041

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA

the specification of which: (check one)

☐ is attached hereto.

☒ was filed on **PCT/EP00/07207**, as United States Application No. or PCT International Application No. **July 26, 2000**
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent Office all information which is known to me to be material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code Section 119 or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Day/Month/Year Filed	Priority Not Claimed
			☐
			☐
			☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

Application Serial No.	**Filing Date**
99115501.1	August 5, 1999

I hereby claim the benefit under 35 U.S.C. Section 120 of any United States application(s), or Section 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C.F.R., Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	**Filing Date**	**Status (patented, pending, abandoned)**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint the practitioners at customer number: 29157

as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and direct that all correspondence be forwarded to:

Bell, Boyd & Lloyd LLC
P.O. Box 1135
Chicago, Illinois 60690-1135

And all telephone calls be directed to: (312) 807-4204.

Full name of sole or first inventor **HARALD BRUESSOW**
Sole or first inventor's signature Harald Bruessow — Date 12th February 2002
Residence 1814 La Tour-de Peilz, Switzerland CHX
Citizenship Switzerland
Post Office Address Ch. de la Chaumeny 13
1814 La Tour-de Peilz, Switzerland

Full name of second inventor **ROBERTO RENIERO**
Second inventor's signature — Date 15/2/2002
Residence 1801 Le Mont-Pelerin CHX
Citizenship Switzerland
Post Office Address 24, Ch. Paudille
1801 Le Mont-Pelerin, Switzerland

Full name of third inventor **~~JOSETTE SIDOTI~~**
Third inventor's signature Josette Sidoti — Date 12.02.02
Residence 1094 Paudex, Switzerland CHX
Citizenship Switzerland
Post Office Address Route du Lac 24
1094 Paudex, Switzerland